Journal of Medical Virology 53:225–228 (1997)

High Prevalence of GBV-C Hepatitis G Virus Infection in a Rural South African Population

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A novel virus, GBV-C/hepatitis G virus (GBV-C/HGV), has been cloned and characterised recently. GBV-C/HGV global epidemiology and risk factors for acquisition are currently unclear. We aimed to establish the determinants of this infection in a rural South African (SA) population. The study population included two samples, namely a community-based sample, and consenting persons from a nonspecialist outpatient department in the same district. A questionnaire regarding demographic details and putative risk factors was administered; blood samples were taken on which a polymerase chain reaction (PCR) was performed for both 5′NCR and NS5a regions of GBV-C/HGV using commercially available primers and probes. Two hundred and forty-nine people were studied with a mean GBV-C/HGV prevalence of 10.4%. Outpatient department and community prevalences differed significantly (18.0% and 6.3%, respectively, P = 0.004). GBV-C/HGV infection was associated with excessive alcohol consumption (P = 0.02; OR, 4.18) and a lack of waterborne sewerage (P = 0.04). PCR amplification of the NS5a region of all but two South African GBV-C/HGV positive samples showed poor reactivity. The prevalence of GBV-C/HGV in rural SA appears to be higher than that reported from Europe and North America. Infection appeared to be associated with excess alcohol intake and a history of previous blood transfusions. The discrepant NS5a and 5′NCR PCR sensitivity in this study raises the possibility of genetic differences in southern African GBV-C/HGV. ***J. Med. Virol. 53:225–228, 1997.***

KEY WORDS: GBV-C; HGV; South Africa; prevalence; RT-PCR

INTRODUCTION

A novel flavivirus, GB virus-C or hepatitis G virus (GBV-C/HGV), has been cloned and characterised recently as distinct from hepatitis C virus (HCV) [Simons et al., 1995; Leary et al., 1996; Linnen et al., 1996]. The global epidemiology of GBV-C/HGV is currently undefined. Those at high risk of parental exposure to infected blood, such as haemophiliacs and intravenous drug addicts, have a high prevalence of GBV-C/HGV [Alter, 1996; Linnen et al., 1996], while the USA and European volunteer blood donor prevalence is approximately 1.5–2.5% [Dawson et al., 1996; Linnen et al., 1996]. A recent report has shown the prevalence of GBV-C/HGV in 290 randomly selected Ghanaian school children to be 14.2% [Dawson et al., 1996] and to increase with age. However, there is currently no data on this infection in adults in Africa.

Although there may be an association between GBV-C/HGV infection and fulminant non-ABC hepatitis [Yoshiba et al., 1995; Heringlake et al., 1996], there is conflicting data as to whether GBV-C/HGV causes active liver disease in all but a very small group of infected individuals [Alter, 1996; Linnen et al., 1996]. It is possible that GBV-C/HGV is benign or is linked to an as yet unidentified disease. Recently, GBV-C/HGV sequences have been detected in saliva [Chen et al., 1997]. Vertical transmission from mothers to infants may occur [Feucht et al., 1996; Moaven et al., 1996].

No commercial anti-GBV-C/HGV antibody assays sensitive for infection are currently available, and reliable diagnosis of this infection is possible only by reverse transcription polymerase chain reaction (PCR). Primers and capture probes, designed according to consensus sequences for both the 5-prime noncoding region (5′NCR) and NS5a region, have been combined in the Hepatitis G Virus Primer-Probe set for GBV-C/HGV diagnosis (Boehringer Mannheim, GmbH Biochemica, Mannheim, Germany) [Schlueter et al., 1996]. PCR amplification of both regions using these primers and probes for each patient sample has been shown to be more sensitive than amplification of one region alone [Schlueter et al., 1996].

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Accepted 13 June 1997

Our aim was to evaluate the prevalence of GBV-C/HGV in a rural district of South Africa (SA), to identify factors associated with infection, and to investigate whether any difference in prevalence existed between a group of adults attending a nonspecialist outpatient department (OPD) clinic and a community sample of adults in the same region.

MATERIALS AND METHODS

Sampling

This study was approved by the University of Cape Town Faculty of Medicine Ethics and Research Committee. Consenting persons were recruited from the rural district of Keiskammahoek (KKH) in the Eastern Cape Province of SA. Recruits lived in formal housing within villages or as subsistence farmers in isolated clusters of traditional mud houses. All recruits were resident in KKH for at least 5 years, 18 years or older, and ambulant. There were two sampling procedures. A random community-based sampling of 160 persons was performed. Four sampling regions were chosen in the KKH district by random number chart. Within these regions, a plot of approximately 2000m^2 in size (independent of the number of houses) was considered to be a sampling unit. Units were randomly sampled until 40 people from each district were recruited. Eligible consenting occupants of each unit were numbered, and a maximum of two were randomly chosen for inclusion. A separate group of 89 sequential adults attending the KKH hospital OPD were recruited. Blood samples were taken and separated within 6 hours and the serum and plasma immediately stored at −20°C (followed by storage at −70°C later).

Evaluation

A questionnaire was completed for each person recruited to the study. This elicited age, gender, residence type (brick, corrugated iron, or mud), source of water (tap or river), mode of sewerage disposal, employment, history of hepatitis or any form of liver disease, past transfusions, scarification (i.e., ritual scarring), and a history of significant trauma requiring hospitalisation. Patients attending the OPD were also questioned about the quantity of alcohol they consumed and their use of drugs. For the purposes of this study, habitual consumption of >60g of alcohol per day or >360g alcohol per weekend was considered excessive [Grønbæk et al., 1994; Becker et al., 1996]. Data on alcohol consumption could not be collected from the community group because of resistance on the part of community leaders that was voiced at the time we were negotiating access to the community.

Reagents and Control Plasmas

Unless otherwise stated, all reagents and control plasmas used in this study were supplied by Boehringer Mannheim, GmbH Biochemica, Mannheim, Germany. The controls supplied by Boehringer Mannheim included GBV-C/HGV RNA negative, low positive, and high positive samples.

TABLE I. PCR Primers and Capture Probes Used for the Amplification and Detection of GBV-C/HGV RNA

	Sequences
5′NCR forward	5′-CGGCCAAAAGGTGGTGGATG-3′
5′ NCR reverse	5′CGACGAGCCTGACGTCGGG-3′
5′NCR capture probe	5′-Biotin-GGTAGCCACTATAGGTGGG-3′
NS5a forward	5′-CTCTTTGTGGTAGTAGCCGAGAGAT-3′
NS5a reverse	5′-CGAATGAGTCAGAGGACGGGGTAT-3′
NS5a capture probe	5′-Biotin-GTTACTGAGAGCAGCTCAGAT-3′

RNA Extraction and cDNA Synthesis

RNA was extracted from 200μl serum or plasma using Total RNA Isolation Reagent (Advanced Biotechnologies Ltd., London, UK) according to the manufacturer's instructions. The RNA was precipitated in isopropanol overnight at −20°C, pelleted, washed with 75% ethanol, and resuspended in 20μl diethyl pyrocarbonate treated ultra pure water. A cDNA synthesis step was performed in a 20μl solution containing 10μl of the extracted RNA with final concentrations of 50nM random hexamers, 200μM PCR nucleotide mix, 1 U RNase inhibitor, 1X RT buffer [50mM Tris-HCl, 8mM $MgCl_2$, 30mM KCl, 1mM dithioerythritol (pH 8.5)], and 10 U Maloney murine leukemia virus reverse transcriptase. This was incubated for 10 minutes at room temperature, 30 minutes at 42°C, and 5 minutes at 95°C.

HGV/GBV-C PCR

PCR was carried out independently for both the 5′NCR and NS5a regions as previously described [Schlueter et al., 1996], with minor modifications. In brief, 5μl of the cDNA solution was added to a 45μl mastermix containing (final concentrations) 1X PCR digoxigenin (dig) labeling mix (200μM dATP, dCTP, dGTP each; 190μM dTTP, 10μM DIG-11-dUTP), 200nM of both forward and reverse primers, 2.6 U Expand High Fidelity polymerase, and 1X PCR buffer [10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$ (pH 8.3)]. Samples were subjected to 40 amplification cycles with three temperature settings of 94°C, 55°C, and 72°C each for 45 seconds, followed by a 7 minute elongation period at 72°C. The primers and capture probes used in this study for both regions were those previously described [Linnen et al., 1996; Schlueter et al., 1996], and provided in the Hepatitis G Virus Primer and Capture Probe set (Table I).

Detection of Amplification Products

Detection of the amplification products was performed with the PCR ELISA DIG Detection system using a streptavidin-coated microtitre plate format. The

amplification products (20μl) were diluted 1:3 with denaturation solution (50mM NaOH) and incubated for ten minutes. Hybridisation buffer (440μl) containing 75ng/ml of the relevant biotinylated probe was added and 200μl of this solution pipetted into microtitre plate wells. The plates were incubated for 180 minutes at 42°C and then washed five times. The provided anti-digoxigenin-peroxidase conjugate in Tris-HCl buffer (pH 7.5) was added to the plates and incubated at 37°C for 30 minutes and then washed 5 times. The enzyme substrate [1.9mM, 2,2′ azino-di(3-ethylbenzthiazoline sulfonate), diammonium salt; ABTS] in 100mM phosphate citrate buffer (pH 4.4)-3.2 mM H_2O_2 (as sodium perborate) was added and the colour developed for 30 minutes at 37°C. The absorbance was measured at 405nm and samples considered positive if the sample OD was >3 times the negative control OD. Samples were considered positive if detected by both 5′-NCR and NS5a primer pairs. If only one PCR was reactive, the sample was considered positive if it was repeatedly reactive.

Statistical Methods

Differences in prevalence were analysed using the chi squared test. Discriminant analysis by stepwise method assessed factors associated with increased risk of infection. Stratified analysis was used to assess the interdependence of risk factors. Behrens-Fisher's t-test was used to compare the means of two sets of numbers. A *P*-value of <0.05 was considered to indicate statistical significance.

RESULTS

A cohort of 249 people were studied, of whom 160 (64.3%) were recruited from the community sampling and 89 (35.7%) from the OPD. The mean age of both the sample populations was 47 years (range, 18–82), and the male:female ratio of the community and OPD groups was 1:2 and 1:1.2, respectively. All members of the community sampling group lived in traditional mud houses with no access to waterborne sewerage. Thirty percent of the OPD sample group lived in formal housing or corrugated iron houses. Of these, 56% had access to waterborne sewerage. No significant differences were shown between the two groups with regard to a history of blood transfusions (10.1% vs. 14.4%) or scarification (73.0% vs. 73.8%). However, more people from the OPD group gave a history of major trauma (27.0% vs. 11.3%; $P = 0.002$), and hepatitis or other liver disease (10.1% vs. 0.63%; $P = 0.0003$).

PCR amplification was performed independently for the 5′NCR and NS5a regions on all samples. An interesting difference was observed between the two sets of results. Twenty-six of the 249 samples were positive by either 5′NCR or NS5a PCR (or both), giving an overall GBV-C/HGV prevalence of 10.4%. However, 23 of these samples were positive by 5′NCR PCR alone, two were positive by both PCRs, and one sample was positive by NS5a PCR only. Both PCR analyses were performed on the same cDNA. Subsequent reanalysis of the same cDNA for samples with discrepant results confirmed positivity.

For the samples found to be positive by 5′NCR but negative by NS5a PCR, a Behrens-Fisher analysis was performed on the NS5a OD readings to assess whether the NS5a reactions were truly negative, or if they were of extremely low efficiency. Although the OD readings for the positive samples were below the manufacturer's cutoff level, statistical analysis showed them to be significantly higher than the negative samples ($P = 0.0097$), suggesting that the cDNA amplification of these samples was of very low efficiency, rather than there being no amplification at all. This may have been due to poor binding of one (or both) of the NS5a primers or of the probe.

Community and OPD prevalences differed significantly with rates of 6.3% (10/160) and 18.0% (15/89), respectively ($P = 0.004$: [OR, 3.29; $1.33 < OR > 8.25$]). Univariate analysis showed a relationship between HGV/GBV-C infection and excessive alcohol consumption in the OPD population ($P = 0.014$: [OR, 4.27; $1.00 < OR > 16.89$]) and a lack of waterborne sewerage ($P = 0.04$; [OR, 4.08; $0.98 < OR > 15.63$]). However, this analysis showed no relationship between infection and age, gender, residence type, water source, employment, a history of hepatitis or any form of liver disease, past blood transfusions, scarification, major trauma, or marijuana usage. None of the recruits gave a history of intravenous drug abuse. This is in keeping with previous reports from SA showing there to be very little current abuse of such drugs [Karassellos and Wilson, 1993]. Discriminant analysis showed excessive alcohol consumption (0.802), female gender (0.505), past blood transfusions (0.432), a lack of waterborne sewerage (0.235), and increasing age (0.232) to be predictors of GBV-C/HGV positivity (standardised coefficients in brackets).

There was no association between the histories obtained regarding alcohol consumption and past transfusions, previous hepatitis, or any form of liver disease, suggesting that each of these risk factors is independent of the others. Stratified analysis confirmed the independence of alcohol consumption, past transfusions, and GBV-C/HGV infection, but too few persons with a history of liver disease or hepatitis were included to permit us to analyse this by stratified analysis. When recruits who had received blood transfusions were removed from both groups and those known to consume excess alcohol were removed from the hospital OPD group, the previously observed difference in the prevalence of GBV-C/HGV between the hospital and community groups was no longer observed ($P = 0.07$).

DISCUSSION

The data show the prevalence of GBV-C/HGV in a South African rural community to be 10.4%. This is far higher than that seen in the United States and Europe [Linnen et al., 1996; Dawson et al., 1996]. The significant difference between OPD patients and those recruited in the community sampling ($P = 0.004$) posed

the question as to whether there is an as yet unrecognised association between GBV-C/HGV infection and other medical conditions. However, when patients consuming excess alcohol and those who had had transfusions were removed from the hospital group, and those who had had previous transfusions were removed from the community group, the prevalence of the infection between the two groups appeared to be similar. Studies investigating risk factors associated with diseases are generally based on case-controlled studies and not cross-sectional stratified samples. However, despite this statistical limitation, evidence was still found in support of several factors being associated with infection.

Our data suggest an association between GBV-C/HGV infection and excessive alcohol ingestion, transfusions, and female gender. The association with alcohol is in keeping with the published HCV data but has not been previously shown in a population-based study of GBV-C/HGV. It is interesting to note that the associations between GBV-C/HGV infection and alcohol and past transfusions are independent of each other and appear to be independent of a history of any form of liver disease. The association with a lack of waterborne sewerage is not in keeping with a virus known to be transmitted parentally but may account for the large numbers of infected individuals without known risk factors for GBV-C/HGV acquisition.

Primers and probes used in this study to detect GBV-C/HGV were designed according to consensus sequence data generated from variants outside of Africa [Schleuter et al., 1995]. The difference between the 5′NCR and the NS5a results in this study suggest alterations in nucleotide sequence which are of diagnostic significance.

ACKNOWLEDGMENTS

We thank Dr. A.L. Williamson of the University of Cape Town Department of Virology for assistance with the preparation of this manuscript. This study was performed in the laboratories of the University of Cape Town Department of Virology. We thank Boehringer Mannheim for their support in providing the reagents for this study.

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